MODELING, SYNTHESIS AND IN VITRO SCREENING OF UNUSUAL AMINO ACIDS AND PEPTIDES AS PROTEASE INHIBITORS

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ABSTRACT

Currently non-proteinogenic amino acids and synthetic peptides are widely used as building blocks in the drugs design. Many of these compounds are enzyme inhibitors or antimicrobials. Secreted Clostridium histolyticum collagenase is considered as a virulence factor and thus is an attractive target for fighting microbial infection. Trypsin is a target for the treatment of digestive disorders. This study was aimed to find new inhibitors of collagenase and trypsin. The interaction of non-proteinogenic amino acids and peptides with C. histolyticum collagenase and trypsin has been evaluated by Molecular Docking followed by the measurement of enzyme inhibition by selected compounds. According to the Docking analysis, N-tert-butoxycarbonylglycyl-(S)-2-amino-3-(4-((4-fluorophenyl)-ethynyl)-phenyl)-α-alanine has to demonstrate the most effective interaction with collagenase. Thus, the synthesis of this dipeptide was carried out. Measurement of enzymes activity revealed that both the dipeptide and the amino acid included in its structure inhibit collagenase and trypsin. The amino acid inhibited collagenase with IC_{50} = 0.32 mM and trypsin with IC_{50} = 0.57 mM. The dipeptide inhibited collagenase with IC_{50} = 0.62 mM and trypsin with IC_{50} = 89 uM.

Keywords: bacterial collagenase, molecular docking, inhibitor, peptide synthesis.

INTRODUCTION

Enantiomerically enriched non-proteinogenic amino acids are valuable compounds for drug design mainly due to their unique structure and two functional groups that are easily modified. Their introduction into peptides structure expands the search for essencial pharmacological agents. Many of these compounds appeared to be enzymes inhibitors or antimicrobial drugs. The majority of pharmacological targets (about 85 %) are proteins, mainly ion channels and enzymes [1]. The collagenases play a key role in remodeling and degradation of extracellular matrix. The imbalance of the activation and inhibition of collagenases is responsible in progression or inhibition of several diseases [2]. Inhibition of collagenase and elastase prevents the loss of skin elasticity induced by photoageing [3]. It was established that inhibitors of clostridial collagenases act as antivirulence agents [4, 5].

Trypsin plays an important role in regulation of digestion process. Upregulation of trypsin activity causes pancreatitis and related diseases which are commonly treated by trypsin inhibitors. Different classes of collagenase and trypsin inhibitors were developed and tested, however inhibitors targeting these enzymes with high selectivity are still required.

Molecular docking approach is used to predict the interaction between non-protein amino acids and peptides with collagenase and trypsin. The inhibition of these enzymes by selected compounds was investigated by in vitro tests.

In this study the interaction of non-protein amino acids and peptides with collagenase and trypsin has been evaluated by Molecular Docking followed by selection
of the most effective compounds. The ability of the prospective compounds to inhibit these enzymes has been investigated.

EXPERIMENTAL

Molecular docking

Structure of compounds were built by ChemBioOffice 2010 (ChemBio3D Ultra12.0). Ligand free energy was minimized using MM2 force field and truncated Newton-Raphson method. Crystallographic structure of collagenase G was taken from the Protein Data Bank of Research Collaboratory for Structural Bioinformatics (PDB ID: 2Y50). Water molecules were removed and polar hydrogens were added. Docking of ligand to enzyme was done by using AutoGrid 4, AutoDock Vina softwares [6]. The ligands were ranked using an energy-based scoring function and a grid-based protein-ligand interaction was used to speedup the score calculation. The most promising interaction models were chosen and hydrogen bounds lengths were measured for them.

Chemistry

The synthesis of dipeptide N-tert-butoxycarbonylglycyl-(S)-2-amino-3-(4-((4-fluorophenyl)-ethynyl)phenyl)-α-alanine has been performed as follows (Fig. 1). At the first stage protected amino acid N-tert-butoxycarboxylglycine (N-Boc-Gly) was synthesized (3) by using glycine (Alfa Aesar CAS No.: 56-40-6) (1) as a starting substance and di-tert-butylpyrocarbonate ([((CH₃)₃CO])₂O) (Acros CAS No.: 24424-99-5) (2) dissolved in i-propanol. Conversion of the N-Boc-Gly (3) to succinimide ester was done according to the scheme presented in Fig. 1. In this reaction N-hydroxysuccinimide (HOSu) (4) (MERCK CAS No.: 6066-82-6) and dicyclohexylcarbodiimide (DCC) (MERCK CAS No.: 538-75-0) were used as water splitting agents, and 1,4-dioxane (Acros CAS No.: 123-91-1) and methylene chloride (MERCK CAS No.: 75-09-2) were used as solvents. The reaction proceeded according to a previously developed methods [7, 8]. The stable N-Boc-Gly-N-oxysuccinimide ester (5) was obtained as a result of the reaction. At the next stage, the

<table>
<thead>
<tr>
<th>Protected amino acid</th>
<th>Non-protein amino acid</th>
<th>Dipeptide</th>
<th>Chemical yield, %</th>
<th>Enantiomeric yield, %</th>
</tr>
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<tbody>
<tr>
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<td><img src="image2.png" alt="Non-protein amino acid" /></td>
<td><img src="image3.png" alt="Dipeptide" /></td>
<td>70</td>
<td>95</td>
</tr>
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Fig. 1. Scheme and yealds of synthesis of N-tert-butoxycarbonylglycyl -(S)-β-4-(4-fluorophenyl)-ethynyl)-phenyl)-α-alanine dipeptide.
condensation reaction of the activated ester of N-Boc-Gly with (S)-β-4-(4-fluorophenyl-ethynyl)-phenyl-α-alanine (6) (previously synthesized in the laboratory for the synthesis of amino acids and peptides) was carried out. This reaction proceeded in the presence of 0.5 N aqueous NaOH (Acros CAS No.:1310-73-2) in 1,4-dioxane at room temperature. As a result, N-Boc-Gly-(S)-β-4-(4-fluorophenyl-ethynyl)-phenyl-α-alanine (7) dipeptide was synthesized.

Materials

All reagents were purchased from corresponding firms and used without further purification. Thin-layer chromatography (TLC) was carried out on Merck aluminium foil backed sheets precoated with 0.2 mm Kieselgel 60 F254. Melting points (mp) were determined by “Elektrothermal”. 1H spectra were recorded on “Varian Mercury 30000 300 MHz spectrometer using TMS as internal standard. The NMR spectra were calibrated by solvent at 7.27 (CDCl3), 3.31 (CD3OD), 4.79 (D2O), 2.50 ((CD3)2SO). The enantiomeric purity of the dipeptide was determined by HPLC (“Waters Alliance 2695 HPLC System”) on a chiral phase of the Diaspher 110-Chirasel-E_1 6 m, 4.0 x 250 mm, mobile phase consisted of methanol:sodium phosphate monobasic buffer (20:80). Enantiomeric yield was proved by chiral HPLC analysis of the isolated amino acids.

Synthesis of N-Boc-Gly

0.247 g (0.0033 mol) of glycine was added to 0.35 mL of NaOH (0.5 M aqueous solution) in a flat-bottomed flask, then 0.185 g (0.0022 mol) of NaCl (Acros CAS No.:7647-14-5) were added to 0.35 mL of NaOH and 0.053 g (0.00063 mol) of NaHCO3 were added to the reaction mixture, stirred for ~ 2 h at 0°C and 1 h at room temperature. The formed precipitate was filtered off, the solvent distilled off on a rotary evaporator and crystallized in a mixture of ethyl acetate : hexane (1:2). Yield of the end product was 75 % (0.469 g).

Synthesis of N-Noc-Gly-succinimide ester (3)

0.291 g (0.0025 mol) of N-hydroxysuccinimide (2) dissolved in a mixture of 6 mL of 1,4 - dioxane and 3 mL of methylene chloride were added to 0.411 g (0.0023 mol) of N-Boc-Gly (1). 0.495 g (0.0024 mol) of DCC dissolved in 3 mL of 1,4 - dioxane was added to the reaction mixture, stirred for ~ 2 h at 0°C and 1 h at room temperature. The formed precipitate was filtered off, the solvent distilled off on a rotary evaporator and crystallized in a mixture of ethyl acetate : hexane (1:2). 0.538 g (0.0019 mol) of (S)-β-4-(4-fluorophenyl-ethynyl)-phenyl-α-alanine in 3 mL of 1,4 - dioxane was placed in a flat - bottom flask and heated to 60°C. Then 6.75 mL of 0.5 M NaOH and 0.053 g (0.00063 mol) of NaHCO3 were added to the reaction mixture. 0.463 g (0.0017 mol) of N-Boc-Gly-N-oxysuccinimide ester dissolved in 2 mL of 1,4 - dioxane was added 15 min later. The reaction mixture was stirred for 3 h at room temperature and kept at 5°C. The next day, 5 mL of ethyl acetate, 3 mL of 10 % citric acid solution and 0.2 g of NaCl (Acros CAS No.:7647-14-5) were added to the reaction mixture and stirred for 15 min. The formed organic layer was separated, dried by Na2SO4 (Acros CAS No.:7757-82-6) and the solvent was removed under vacuum at 50°C. The residue was recrystallized in ethyl acetate-hexane mixture (1:3). The reaction was monitored by thin-layer chromatography in the system of chloroform (MERCK CAS No.:67-66-3) : methanol (MERCK CAS No.: 67-56-1) : ethyl acetate (3:2:1). The yield was 70 % (0.52 g). Found, %: C 65.36; H 5.93; N 5.97. C28H28FN2O5 Calculated, %: C 65.44; H 5.72; N 6.36. 1H NMR (300 MHz, DMSO) δ = 1.42 (9H, s, t-C3H7); 2.97 (1H, dd, J = 13.6, J = 7.8) and 3.12 (1H, dd, J = 13.6, J = 9.6).
Determination of collagenase activity

Collagenase activity was measured by the method based on determining free amino groups released as a result of substrate hydrolysis [10]. The reaction mixture contained 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (MERCK CAS No.: 7365-45-9), pH 7.2, 10 mg mL⁻¹ gelatin (MERCK CAS No.: 9000-70-8) and 0.025 mg mL⁻¹ collagenase (Alfa Aesar CAS No.: 9001-12-1). The reaction proceeded at 37°C. The concentration of amino groups in the reaction mixture was determined by ortho-phthalaldehyde (OPA) reagent containing 0.2 M borate buffer, pH 9.7, 0.1667 mg mL⁻¹ OPA (Alfa Aesar CAS No.: 643-79-8) and 1.18 mM mercaptoethanol (Alfa Aesar CAS No.: 60-24-2). The aliquot (50 μL) was picked up every 30 min. The reaction was stopped by addition of 6 μL of 30 % trichloroacetic acid. OPA reagent (1.5 mL) and H₂O (1.5 mL) were added to aliquot and A₃₄₀ was recorded after 5 min incubation at 27°C.

Determination of trypsin activity

Trypsin activity was determined according to the method recommended by “Millipore” Corporation [11]. The reaction mixture contained 46 mM Tris-HCl buffer, pH 8.2 with 11.5 mM calcium chloride, 0.01 M N-β-Tosyl-L-Arg-OMexHCl (TAME) (MERCK CAS No.: 1784-03-8). After incubation and temperature equilibration (3 - 4 minutes to achieve 25°C), 12 μg mL⁻¹ trypsin (MERCK CAS No.: 9002-07-7) was added (0.1 mL of diluted enzyme). A₂₄₇, was recorded for 3 - 4 minutes. ΔA₂₄₇ was determined from initial linear portion of the curve. The reaction remained linear to an A₂₄₇ of about 0.320 [12].

RESULTS AND DISCUSSION

Docking of collagenase with non-protein amino acids synthesized earlier has been done. Inhibition of collagenase by the compounds that demonstrated the lowest ΔG (< -7.0 kcal mol⁻¹) was studied [13]. According to the results, (S)-2-amino-3-(4-((4-fluorophenyl)ethynyl)phenyl)-α-alanine (6) (ΔG - 7.5 kcal mol⁻¹) turned out to be the strongest inhibitor compared to the rest of studied compounds. The IC₅₀ for this compound was estimated to be 0.32 mM.

In this study, models of peptides containing 6 have been constructed and docking of these peptides with collagenase has been performed. According to the Docking data, dipeptide N-tert-butoxycarbonylglycyl-(S)-2-amino-3-(4-((4-fluorophenyl)ethyl)phenyl)-α-alanine (7) demonstrated appropriate characteristics (ΔG -7.8 kcal mol⁻¹). Both compounds 6 and 7 bound collagenase symmetrically at the same site/pocket of catalytic subdomain (Fig. 2) [14]. The dipeptide (7) inhibited...
collagenase with estimated $IC_{50} = 0.62$ mM.

The influence of 6 and 7 on the activity of trypsin has been also studied. Both compounds inhibited trypsin activity with estimated $IC_{50}$ values of 89 uM for dipeptide (7) and 0.57 mM for amino acid (6). Thus, compounds (6 and 7) demonstrated the ability to inhibit both C. histolyticum collagenase (metalloprotease) and trypsin (serine protease).

**CONCLUSIONS**

New inhibitors of clostridial collagenase have been revealed in this study: (S)-2-amino-3-(4-((4-fluorophenyl)-ethynyl)-phenyl)-α-alanine (6) ($IC_{50} = 0.32$ mM) and dipeptide N-tert-butoxycarbonyl-glycyl-(S)-2-amino-3-(4-((4-fluorophenyl)-ethynyl)-phenyl)-α-alanine (7) ($IC_{50} = 0.62$ mM).

According to the results, inhibition of collagenase by dipeptide was slightly decreased. On the other hand, the solubility of dipeptide (7) in H$_2$O was significantly higher compared to that of amino acid (6), which makes dipeptide more suitable for further application (data not shown). Both compounds inhibited trypsin activity. Estimated $IC_{50}$ value of 89 uM for dipeptide (7) suggests that this compound is a strong inhibitor of trypsin.

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